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<b>13. SUPPLEMENTARY NOTES</b>						
<p><b>14. ABSTRACT:</b> The purpose of the project is to characterize the breast cancer in women affected with Neurofibromatosis type 1 (NF1) in a multi-institutional setting. <u>Aim 1</u> assessed the incidence of breast cancer in this cohort and the clinical features of NF1 associated with breast cancer and other cancers. A total of 423 cases of NF1 women have been reviewed. History of breast cancer was found in 20. Family history of cancer or breast cancer is associated with a personal history of breast cancer. Neither cutaneous neurofibroma burden nor family history of NF1 was found to be associated with the occurrence of breast cancer in this study. Malignant peripheral nerve sheath tumor (MPNST) is associated with plexiform neurofibromas. Learning disability is associated with CNS tumor and/or optic glioma (OPG). European Americans (EA) are more likely to develop CNS tumor and/or OPG than African Americans (AA). <u>Aim 2</u> investigated the NF1 gene germline mutations in women with breast cancer. Germline NF1 mutation has been investigated for 14 women. The types of mutations were not significantly different from the general NF1 population. Germline exome sequencing (WES) has been completed for 14 NF1 women with history of breast cancer and female 42 controls. Preliminary WES data analysis has not identified any deleterious mutations in high penetrance risk genes for breast cancer. <u>Aim 3</u> completed the IHC analysis for signaling pathways and growth factor receptors. Nine out of 14 breast cancers from women with NF1 are found to be HER2/NEU positive, the portion of which is significantly higher than the general population breast cancers. The tumor specimen genome, copy number, and methylation analysis is to be completed. <u>Aim 4</u>. NF1 inactivation results in human mammary epithelial cells (HMEC) senescence; p53 inactivation does not rescue the senescence phenotype in NF1KD (knockdown) HMEC; p53 inactivation provides an initial growth advantage to HMEC with a consequent large</p>						
<b>15. SUBJECT TERMS</b> NF1; breast cancer; signaling pathway; germline mutation; somatic mutation; FFPE; IHC; next generation sequencing, NF1 knockdown cells; senescence, Ras						
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## 1. INTRODUCTION

The occurrence of breast cancer is increased in women affected with Neurofibromatosis type 1 (NF1). This study is aimed at identifying an accurate incidence of breast cancer in this group of women in a multi-center collaborative environment. There are 4 specific aims. **Aim 1** is to confirm the increased breast cancer risk in women with NF1. All the participating centers, Henry Ford Health System (HFHS), University of Alabama at Birmingham (UAB), Children's National Medical Center in D.C. (CNMC), and Johns Hopkins University (JHU), have reviewed the medical records of women affected with NF1. Clinical data were analyzed to identify clinical features associated with the occurrence of breast cancer. Clinical features were also analyzed for their association with other type of cancers in this study. At the same time, women with a history of breast cancer were recruited to donate blood and their archived tumor specimen. **Aim 2** is to analyze the germline *NF1* gene and whole exome in the subjects with history of breast cancer. The NF1 mutations identified were analyzed for genotype-breast cancer correlation. Additional germline gene changes may reveal breast cancer predisposition in addition to the NF1 gene. **Aim 3** is to determine if NF1 associated breast cancers have unique characteristic signaling pathways or molecular tumorigenesis. Immunohistochemistry study of the signaling pathways was performed on archived tumor blocks. NF1 gene mutation, whole exome sequence, copy number variation and DNA methylation will be analyzed on these tumor specimens. **Aim 4** is to study the phenotype of NF1 knockdown in primary mammary epithelial cells, specifically focused on the senescence effect due to Ras activation. This study attempts to provide information in determining when and how to screen for breast cancer in this group of women. It will also shed light on the molecular mechanisms of breast cancer in NF1 deficient human subjects.

## **2. KEY WORDS**

Neurofibromatosis type 1; breast cancer; signaling pathway; germline mutation; somatic mutation; FFPE; IHC; whole exome sequencing, NF1 knockdown cells; senescence, Ras

### 3. ACCOMPLISHMENTS

- What were the major goals and what was accomplished under these goals?

**Aim 1: To confirm the increased breast cancer risk in women with NF1. To identify any clinical features associated with the risk for breast cancer.**

**Task 2:** Clinical data collection, analysis, patient contact and specimen retrieval

**2a.** Chart review and data recording in each clinical site -- 423 cases collected.

-- Completed (Dec 2013)

**2b.** Aim 1 data analysis in HFHS

-- Completed (Sept 2015)

**2c.** Obtain consent, archived tumor specimens and blood, obtain previous genetic testing results.

--Completed (May 2014)

**2d.** Recruit more breast cancer cases outside NF clinics, obtain record, archived tumor specimens and blood.

-- Completed (May 2014)

**2e.** Manuscript development for Aim 1

-- Completed (Oct 2015)

In this reporting period, thorough and comprehensive analysis has been completed for the family history, clinical features and cancers in 423 cases of women affected with NF1. Multiple publications have proven that the incidence of breast cancer is higher in women with NF1 especially in women under 50 years of age. At least one type of cancer was reported in 98 women with NF1. Nineteen of them have had at least two primary cancers. There were 205 prevalent cancer/neoplasms in the relatives of 125 women with NF1.

**Breast cancer:** Twenty women have had a diagnosis of breast cancer. Half of the cases (n=10) were diagnosed between the age of 40 to 49 years. A quarter of the cases (n=5) were diagnosed between 30 to 39 years of age. Two cases were diagnosed with a second primary breast cancer.

**Statistical analysis:** We used Fisher's exact tests to evaluate the statistical significance of association between each discrete clinical feature and prevalent cancers. P-values less than 0.05 were considered to be statistically significant. For ease of interpretability, odds ratios (OR) and corresponding 95% confidence intervals were also estimated to provide estimates of effect.

**Breast cancer and family history:** The prevalence of personal history of breast cancer was nearly four-fold higher (odds ratio OR=3.83, 95% confidence interval 95%CI=1.40-11.12) for women with NF1 and a family history of any cancers (9.6%; 12/125) in comparison to those without a family history (2.7%, 8/298), which was statistically significant ( $p=0.004$ ). The prevalence of personal breast cancer in the presence of a family history of breast cancer in 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> degree female relatives (10.7%, 8/75) is more than 3-fold higher (OR=3.46; 95%CI=1.09 – 11.02) than in the absence of a family history (3.3%, 8/241), which is statistically significant ( $p=0.029$ ). However, breast cancer is not significantly associated with family history of female relatives with NF1 ( $p=0.434$ ). In this cohort, none of the women with breast cancer had a reported family history of any relative affected with NF1 and breast cancer.

**Breast Cancer and Clinical Features of NF1:** For cases with available clinical features, statistical analysis has not detected any association between the NF1 features, breast cancer or other cancers (all  $p\geq 0.16$ ). It is noteworthy to mention that high cutaneous neurofibroma burden (20 or more or described as “diffuse” in the medical record) is not significantly associated with any types of cancer ( $p=1.00$ ).

**MPNST and Plexiform Neurofibroma:** The occurrence of MPNST is related to plexiform neurofibromas (PN).

**CNS tumor, optic glioma (OPG) and learning disability:** The prevalence of CNS tumors (excluding OPG) is nearly 6-times higher (OR=5.73, 95%CI=1.54 – 20.13) in women with a history of OPG (14.6%, 6/41) in comparison to the women without OPG (2.9%, 8/278), which was statistically significant (p=0.004). The women with learning disability have a 2.25-fold (95%CI=1.08-4.67) higher rate of CNS tumor, OPG or both (i.e. “CNS+OPG”) (22.2%, 20/90) than those without a learning disability (11.2%, 21/187), which is significant (p= 0.019).

**Ethnicity and Malignant Neoplasms:** The rate of “CNS+OPG” and “Other cancers” varies significantly by ethnicity. “Other cancers” refers to all malignant tumors, hematological malignancies, CNS tumors and OPG, excluding breast cancer. For the “CNS+OPG” category, European Americans (EAs) were 3.72 times (95% CI=1.48 – 11.16) more likely to develop these tumors (21.2%, 41/193) than African Americans (AAs) (6.8%, 6/88), which was statistically significant (p=0.002). The occurrence of OPG with or without CNS tumor is also higher (OR=3.48, 95%CI=1.28 – 11.88, 95%) in EAs (17.4%, 32/184) than AAs (5.7%, 5/88), which was significant (p=0.008).

**The conclusions:** Family history of breast cancer or all cancers may predict the personal risk of breast cancer in women with NF1. Plexiform neurofibroma may be a predictor for MPNST. Learning disability and European ancestry may be a predictor for central nervous system tumor, including optic glioma.

A manuscript has been submitted to the journal, “Clinical Genetics”. Manuscript is attached in the appendices.

The goal has been met for this Aim.

**Aim 2: To analyze germline NF1 gene of the subjects with history of breast cancer. The mutations identified will be analyzed for genotype-phenotype correlation; Germline whole exome sequencing (WES) on blood**

**Task 3: NF1 gene mutation testing and mutation data analysis.**

**3a.** Consent subjects and send blood for clinical germline NF1 gene analysis.

-- Completed (May 2014)

**3b.** NF1 genotype data analysis

-- Completed (June 2014)



3c. Consent, blood collection and whole exome sequencing (WES): -- Completed (May 2014)

3e. Whole exome sequencing data annotation and analysis – 50% completed -- (month 38-50)

3f. Manuscript development for Aim 2 -- 20% completed -- (month 50-51)

3g. Sanger sequencing confirmation for clinical actionable mutations identified by WES – *No clinical actionable mutation identified*

During this reporting period, we have been preparing a manuscript describing the types of **germline mutation in NF1 gene** found in women with a history of breast cancer. The mutational spectrum in our NF1 patients with breast cancer did not differ from the unselected large cohort described by Sabbagh (Chi square, 2- tailed:  $p=0.23$ ,  $p=0.44$ , resp.  $p=0.59$ ). **Table 1.**

**Table 1. *NF1* gene mutation, breast cancer pathology and cancer history of the 14 NF1 patients studied**

	Breast Cancer								Personal History Other Cancer	Family History Cancer	Family History NF1	Female Relative breast cancer and NF1	Mutation (DNA level; RNA level; protein level)		
ID	Age Diagnosis (year s)	Age Men-arche (year s)	Pathology										Exon	Type	Description
			Type	ER	PR	Her2	Ki-67	Proposed subtype							
1	56	13	IDC	NA	NA	NA	NA	NA	--	--	De novo	--	33 [42]	OOF skipping exon 33 [42] - PSC	c.6364G>A; r.6085_6364del; p.Val2029Lysfs*7
2	53	NA	IDC	+	+	--	NA	Luminal A/B	Carcinoid tumor; Pheochromo-cytoma	Breast; Ovary; Esophagus	Inherited	--	10c [14]	Frame-shift - PSC	c.1541_1542delAG; r.1541_1542delag; Gln514Argfs*43
3	60	16	DCIS	+	+	NA	NA	Luminal B	--	Breast; Ovary; Pancreas; gastrum	De novo	--	37 [46]	IF skipping exon 37 [46]	c.6792C>G; r.6757_6858del; p.Ala2253_Lys2286del
4	49	NA	IDC	--	--	+	NA	HER2	Cervical cancer	Breast; Pheochromo-cytoma	Inherited	NA	15 [20]	Non-sense - PSC	c.2398G>T, r.2398g>u; p.Glu800*
5	45	14	NA	NA	NA	NA	NA	NA	--	--	De novo	--	Intron 26 [34]	OOF splicing - PSC	c.4515-20_4515-18delAAG; r.4514_4515ins4515-14_4515-1; p.Arg1505Serfs*53
6	47	11	IDC	+	+	--	NA	Luminal A/B	--	Breast; Prostate	NA	--	16 [21]	Trunca-tion and low level OOF splicing - PSC	c.2621_2634dupAGGGTTCTATGATT; r.2621_2634dupagggguucuaugauu and r.2618_2850del; p.Ser879Argfs*4 and p.Lys874Phefs*4
7	39	NA	IDC	+	+	NA	NA	Luminal A/B	--	--	Inherited	--	29-30 [38-39]	copy number variant - PSC	c.(5045_5337)_(5625_5796)del; r.5206_5749del; p.Gly1737Leufs*3
8	41	11	IDC	+	--	Eq	NA	Luminal A/B	--	Lung; Colon; Pheochromo-cytoma	Inherited	--	12a [16]	Mis-sense	c.1733T>G; r.1733u>g; p.Leu578Arg
9	49	14	IDC	+	--	+	3 (20%)	Luminal B	--	Breast	NA	NA	Intron 31 [40])	OOF splicing - PSC	c.5943+1G>T; r.5901_5943del; p.Met1967Ilefs*10
10	44	NA	IDC	+	+	--	3 (15%)	Luminal B	--	Breast; Colon; Lung; Prostate	Inherited	NA	16 [21]	Frame-shift - PSC	c.2728_2729delGG; r.2728_2729delgg; p.Gly910Thrfs*8
11	58	NA	NA	NA	NA	NA	NA	NA	--	--	NA	NA	28 [37]	Frame-shift -	c.4910_4911delTT; r.4910_4911dely; p.Phe1637Serfs*3

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ID	Age Diag-nosis (year s)	Age Men-arche (year s)	Pathology										Exon	Type	Description
			Type	ER	PR	Her2	Ki-67	Proposed subtype							
													PSC		
12	52	13	IDC	--	--	+	NA	HER2	--	Breast; Gastrium	De novo	--	30 [39]	Frame-shift - PSC	c.5667dupT; r.5667dupu; p.Ile1890Tyrfs*2
13	47	13	IDC	24%	2%	--	59%	Luminal B	--	Breast; Ovary	Inherited	+	9 [11]	deep intronic splice mutation - PSC	c.1260+1604A>G,r.1260_1261ins1260+1605_1260+1646; p.Ser421_Val2818delinsLeuThrThr*
14	42	13	IDC	+	+	--	NA	Luminal A/B	--	Breast; Ovary	Inherited	+	9 [11]	deep intronic splice mutation - PSC	c.1260+1604A>G, r.1260_1261ins1260+1605_1260+1646; p.Ser421_Val2818delinsLeuThrThr*

We had a hypothesis that a pathogenic germline NF1 genetic variant may be an independent risk factor for breast cancer. Based on this assumption, family history of NF1 should be associated with breast cancer in this population. This hypothesis is not supported by the results of this study, however. The work in Aim 1 demonstrates that a personal history of breast cancer in women with NF1 is associated with a family history of breast cancer (OR=3.46) or all cancers (OR=3.83) while it does not appear to be solely dependent on family history of NF1 in female relatives, or perhaps the magnitude of dependence is not strong enough to be manifested in this study. Therefore, the higher prevalence of breast cancer in NF1 is likely a manifestation of synergistic effects when individuals with an NF1 gene mutation encounter environmental carcinogens or other hereditary cancer predisposition genomic variants of moderate or low penetrance.

Germline DNA extracted from 14 NF1 women who was diagnosed with breast cancer underwent **WES analysis**. Germline DNA from 43 women also underwent WES as controls. Preliminary analysis of the germline whole exome sequencing data has not revealed any deleterious mutation in the known high penetrance breast cancer predisposition genes, *BRCA1*, *BRCA2*, *TP53*, *PTEN*, *CDH1*, *STK11* or *PALB2*. Comprehensive analysis for all variants of the whole exome is ongoing.

**Preliminary analysis** was done for the 14 NF1 women with breast cancer. We have utilized the Omicia, Opal Research™ clinical interpretation program for NGS data. It is designed to assess the links between disease and prioritized genes with automated clinical annotations. The program has the following functions:

- Sequencing Quality Assessment – Determine the quality of your genome variant file with the Omicia Clinical Grade.
- Automated Genome Annotation – Attach evidence to every variant with known clinical relevance, drawing annotations from data sources including OMIM, ClinVar, and COSMIC.
- Predicted Pathogenicity Scoring – Assess each variant on the severity of its impact on protein function with 19 scores including SIFT, PolyPhen, CADD, and the Omicia Variant Score.

The Omicia Score is a proprietary score that assesses whether a variant is likely to be deleterious. It is a meta-classifier that combines scores from the following variant scoring algorithms:

- SIFT (Ng et al. 2001)
- PolyPhen (Adzhubei et al. 2010)
- MutationTaster (Schwarz et al. 2010)
- PhyloP (Siepel et al. 2006)

By combining these scores, the Omicia score synthesizes into a single convenient score. The Omicia score ranges from 0 to 1. An Omicia score of less than 0.5 would indicate that a variant is likely benign. On the other hand, scores greater than 0.5 suggest that a variant is likely to be damaging or deleterious, with higher confidence at values closer to 1.

After being filtered based on the variant frequency (MAF from 1000genome database) and Omicia variant score, the following variants are summarized in **Table 2**. Since we have not completed the variant analysis for control samples, we do not know if these variants are also seen in the control samples.

<b>Table 2. Germline variants from NF1 women affected with breast cancer that are possibly significant.</b>				
<b>ID</b>	<b>Rare variant MAF=&lt;1% unless specified</b>	<b>Omicia Score &gt; 0.9, unless specified</b>	<b>Variant info, missense unless specified</b>	<b>Role of the gene</b>
1166	APC			Tumor suppressor protein that acts as an antagonist of the Wnt signaling pathway
2090	EGFR			EGFR is a cell surface protein that binds to epidermal growth factor; activates at least 4 major downstream signaling cascades including the RAS-RAF-MEK-ERK, PI3 kinase-AKT
1752	ERCC6			Transcription-coupled excision repair
955	MAP2K3			MAP kinase-mediated signaling cascade. It phosphorylates and thus activates MAPK14/p38-MAPK
1061	MAP2K3			MAP kinase-mediated signaling cascade. It phosphorylates and thus activates MAPK14/p38-MAPK
1165	MAP2K3			MAP kinase-mediated signaling cascade. It phosphorylates and thus activates MAPK14/p38-MAPK
1166	MAP2K3			MAP kinase-mediated signaling cascade. It phosphorylates and thus activates MAPK14/p38-MAPK
1448	MAP2K3			MAP kinase-mediated signaling cascade. It phosphorylates and thus activates MAPK14/p38-MAPK
1752	MAP2K3			MAP kinase-mediated signaling cascade. It phosphorylates and thus activates MAPK14/p38-MAPK
1958	MAP2K3			MAP kinase-mediated signaling cascade. It phosphorylates and thus activates MAPK14/p38-MAPK
1992	MAP2K3			MAP kinase-mediated signaling cascade. It phosphorylates and thus activates MAPK14/p38-MAPK
2090	MAP2K3			MAP kinase-mediated signaling cascade. It phosphorylates and thus activates MAPK14/p38-MAPK
2398	MAP2K3			MAP kinase-mediated signaling cascade. It phosphorylates and thus activates MAPK14/p38-MAPK
3073	MAP2K3			MAP kinase-mediated signaling cascade. It phosphorylates and thus activates MAPK14/p38-MAPK
3410	MAP2K3			MAP kinase-mediated signaling cascade. It phosphorylates and thus activates MAPK14/p38-MAPK
1165	MSH3			Forms a heterodimer with MSH2 to form MutS beta, part of the post-replicative DNA mismatch repair system
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<b>Table 2. Germline variants from NF1 women affected with breast cancer that are possibly significant.</b>				
<b>ID</b>	<b>Rare variant MAF=&lt;1% unless specified</b>	<b>Omicia Score &gt; 0.9, unless specified</b>	<b>Variant info, missense unless specified</b>	<b>Role of the gene</b>
2398	NOD2	0.8	Frameshift variant; associated with Crohn's disease	Gastrointestinal immunity
3073	NOD2	0.8	Frameshift variant; associated with Crohn's disease	Gastrointestinal immunity
1448	PIGF			Glycosylphosphatidylinositol GPI-anchor; one of its related pathways is VEGF Signaling Pathway
2090	SH3GLB1			SRC homology 3 domain-containing protein; be involved in regulating apoptotic signaling pathways
1448	TACO1			mitochondrial protein that function as a translational activator of mitochondrially-encoded cytochrome c oxidase 1

### **Aim 3: To determine if NF1 associated breast cancers have unique signaling pathway and molecular tumorigenesis characteristics.**

**Task 4:** Tumor specimen molecular analysis by LOH and methylation assay for NF1, p53, BRCA1, BRCA2, PTEN, and ATM genes and IHC assay for proteins, pMEK, ERK, pERK, AKT, mTOR, p53, PTEN, Her2, Ki67 proteins

**4a.** Assay validation for LOH/methylation (by MLPA) and IHC: *Due to the limited amount of DNA extracted from the tumor FFPE material, LOH assay is being changed to the next generation sequencing NGS technology. Methylation analysis will use 450K array technology.*

– 0% completed (month 49-50)

**4b.** LOH/methylation will be carried out by NGS and 450K array methods – 0% completed (month 49-50)

IHC for tumor specimens -- Completed (July, 2015)

**4d.** Data analysis and manuscript development for Aim 3. (month 50-51)

Immunohistochemistry assay has been conducted for p53, mTOR, PTEN, Phospho-MEK 1/2 (Ser221), Phospho-p44/42 MAPK (ERK1/2), P44/42 MAPK (Erk 1/2), AKT (pan), HER2/ERB2. Antibodies were purchased from Cell Signaling Technology.

#### **METHODS:**

**Antibody selection:** All the antibodies were obtained from the Cell Signaling Technology. IHC staining for pMEK (the phosphorylated and activated form of MEK), ERK (the non-phosphorylated ERK) and pERK (the phosphorylated and activated ERK) proteins was performed. To examine PI3K pathway activation, IHC for pAKT (phosphorylated and activated AKT) and mTOR protein was done. Antibodies against p53, PTEN and HER2 proteins were also validated by IHC.

- P53 (75F) Rabbit mAB (Catalog #2527): Nuclear staining
- mTOR (7C10) Rabbit mAB (Catalog #2983): cytoplasmic staining
- Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (D13.14.4E) XP<sup>TM</sup> Rabbit mAB (Catalog #4370): Nuclear
- Phospho-MEK 1/2 (Ser221) (166F8) Rabbit mAB (Catalog #2338)
- PTEN (D4.3) Rabbit mAB (Catalog #9188)
- AKT (pan) (11E7) Rabbit mAB (Catalog #4685)
- P44/42 MAPK (Erk 1/2) (137F5) Rabbit mAB (Catalog #4695)
- HER2/ERB2(29D8) Rabbit mAB (Catalog #2165)

The findings are illustrated in Table 3.

Table 3. Immunohistochemistry analysis for breast cancer in NF1											
Study ID	Surg path #	histology	PHOSPHO-MEK1/2			PHOSPHO-MAPK (Erk 1/2) P44/42			MAPK (Erk 1/2) P44/42		
			Intensity	% positive	H score	Intensity	% positive	H score	Intensity	% positive	H score
OP-1001	S-7762-06-F	IDC Small tumor	0	0	0	0	0	0	1	90	90
1070	S01-6732	DCIS only	1	80	80	3	20	60	1	30	30
OP-1003	S13-8539	IDC	1	100	100	3	20	60	2	65	130
OP-1004	P12-3709	IDC+DCIS	1	100	100	0	0	0	2	90	180
OP-1005	W1203-111	DCIS+IDC?	1	100	100	3	2	6	1	100	100
OP-1006	6198-6B-13	DCIS+IDC	1	50	50	3	5	15	1	95	95
1299	S03-7282	IDC+DCIS	0	0	0	2	30	60	0	0	0
OP-1007	S14-5536	small tumor DCIS+IDC	1	100	100	0	0	0	1	100	100
OP-1008	S-03-21705	DCIS+IDC	1	100	100	3	1	3	1	95	95
HS06-19153	HS06-19153	IDC	1	50	50	0	0	0	1	70	70
HS08-3064	HS08-3064	DCIS	1	100	100	3	3	9	2	100	200
S13-3214-2	S13-3214-3	DCIS	2	100	200	3	70	210	2	100	200
S13-3214-2	S13-3214-3	IDC	2	100	200	3	40	120	2	100	200
S14-5942-2	S14-5942-3	IDC	2	100	200	3	30	90	1	40	40
	S03-7282: ?poor processing leading to false negative results					Sclerosing adensis high p44 expression			S14-5536-1 DCIS 2+ / 95%		
						Infiltrating edge has highp44 expression					
	100 OR LESS looks negative										
	Maximum H score: 300										



Table 3. Immunohistochemistry analysis for breast cancer in NF1																		
Study ID	Surg path #	His-tology	PTEN			AKT			mTOR			p53			HER2/NEU		ER	PR
			Inten-sity	% positive	H score	Inten-sity	% positive	H score	Inten-sity	% positive	H score	Inten-sity	% positive	H score	Inten-sity	POS/NEG		
OP-1001	S-7762-06-F	IDC Small tumor	1	100	100	1	50	50	0	0	0	1	20	20	0	NEG	POS	POS
1070	S01-6732	DCIS only	1	100	100	1	50	50	0	0	0	3	90	270	3	POS	NEG	NEG
OP-1003	S13-8539	IDC	0	0	0	2	100	200	3	40	120	2	80	160	3	POS	POS	POS
OP-1004	P12-3709	IDC+D CIS	1	100	100	1	80	80	1	100	100	1	5	5	1	NEG	POS	POS
OP-1005	W1203-111	DCIS+I DC?	1	100	100	2	100	200	1	100	100	3	95	285	3	POS	POS	NEG
OP-1006	6198-6B-13	DCIS+I DC	1	100	100	1	80	80	1	100	100	3	95	285	3	POS	POS	NEG
1299	S03-7282	IDC+D CIS	0	0	0	0	0	0	0	0	0	0	0	0	0		POS	POS
OP-1007	S14-5536	small t umor DCIS+I DC	1	100	100	2	100	200	0	0	0	0	0	0	1	NEG	POS	POS
OP-1008	S-03-21705	DCIS+I DC	1	100	100	1	100	100	1	90	90	1	5	5	1	NEG	POS	POS
HS06-19153	HS06-19153	IDC			0	1	100	100	1	100	100	2	85	170	3	POS		
HS08-3064	HS08-3064	DCIS	1	100	100	2	95	190	2	100	200	1	30	30	2 TO 3	POS		
S13-3214-2	S03-3214-3	DCIS	1	100	100	1	95	95	2	90	180	3	95	285	3	POS		
S13-3214-2	S13-3214-3	IDC	1	100	100	1	100	100	1	100	100	3	95	285	3	POS		
S14-5942-2	S14-5942-3	IDC	1	100	100	2	100	200	3	90	270	1	2	2	3	POS		
	S03-7282: ?poor processing leading to false negative results		PTEN LOSS in some cases												S13-8539: Heterogenous			
															P12-3709: DCIS sample positive			
	100 OR LESS looks negative														6198-6B-13: ?All DCIS positive			
	Maximum H score: 300																	

Approximately 15-20% of the breast cancers in the general population are HER2/NEU positive. Out of 15 FFPE breast tumor specimens from NF1 women, 9 were stained positive for HER2, which appears to be significantly higher than the general population,  $p=0.0012$ .

Only two tumors were stained positive for PHOSPHO-MAPK (Erk1/2) P44/42, representing the activated MAPK/MEK pathway.

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**Aim 3: To determine if NF1 associated breast cancers have unique signaling pathway and molecular tumorigenesis characteristics.**

*Task 5:* Next generation cancer gene sequencing including NF1, BRCA1, BRCA2, TP53, PTEN, and ATM genes, and additional breast cancer gene targets including; CDH1, RB1, MLL3, MAP3K1, CDKN1B, PIK3CA, AKT1, GATA3, TBX3, RUNX1, CBF, AFF2, PIK3R1, PTPN22, PTPRD, 3F3B1, CCND3 and possibly other genes on the FFPE tissue.

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**5a.** Next generation sequencing on tumor specimens

-- 0% completed (month 49-50)

**5c.** Data analysis and manuscript development for Aim 3.

-- 0% completed (month 51)

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- **What opportunities for training and professional development has the project provided?**  
Nothing to Report
- **How were the results disseminated to communities of interest? Nothing to Report**
- **How were the results disseminated to communities of interest? Nothing to Report**
- **What do you plan to do during the next reporting period to accomplish the goals?** Please refer the details described under each Aim.

## 4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**
  - A. The following findings are likely to be used to guide targeted cancer screening for individuals (especially females) affected with NF1.
    - a. The family history of breast cancer or all cancers may predict the personal risk of breast cancer in women with NF1.
    - b. Plexiform neurofibroma may be a predictor for MPNST.
    - c. Learning disability and European ancestry may be a predictor for central nervous system tumor, including optic glioma.
  - B. At this time, this study does not support the hypothesis that NF1 gene mutation is an independent hereditary risk factor for breast cancer. The moderately elevated risk of breast cancer in women with NF1 is likely a manifestation of the synergistic effects between the NF1 gene mutation, environmental carcinogens and/or other hereditary cancer predisposition genomic variants. When exists independently, these variants are likely of moderate or low risk for cancer.
- **What was the impact on other disciplines?** Nothing to Report
- **What was the impact on technology transfer?** Nothing to Report.
- **What was the impact on society beyond science and technology?** Nothing to Report.

## 5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

Due to the limited amount of DNA extracted from the tumor specimens, as well as the availability of the new DNA analysis technologies, we changed the technological strategies for tumor DNA analysis. In addition, GeneDx has discontinued the Ion torrent Ampliseq service. We were previously contracted with GeneDx for the next generation sequencing tumor specimen and have purchased the primers for the Ampliseq, but now have to abandon this plan. We also abandoned the MLPA method for DNA copy number analysis and methylation analysis due to its demand for higher quantity of DNA.

We now plan to use Agilent OneSeq to accomplish tumor sequencing and copy number variation (LOH –loss of heterozygosity) analysis. We plan to use Illumina 450K array for methylation analysis.

There is no significant impact on the expenditures. All technological changes are planned without needing extra fund.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:** None
- **Significant changes in use or care of human subjects:** None
- **Significant changes in use or care of vertebrate animals:** Does not apply.
- **Significant changes in use of biohazards and/or select agents:** Does not apply.

## **6. PRODUCTS**

- **Publications, conference papers, and presentations:**

Publication has been submitted to Clinical Genetics. A copy is attached in the section of Appendices.

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	<i>Xia Wang MD, PhD</i>
Project Role:	<i>Consultant, previous PI</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	Dr. Wang has been organizing and directing the entire project process towards its completion. She has been responsible for the reporting, communication, budgeting, securing the labs and personnel, and writing the manuscripts for publication.
Funding Support:	<i>Employed in Moffitt Cancer Center</i>

Name:	<i>Dhananjay Chitale, MD, PhD</i>
Project Role:	<i>Current PI, co-investigator</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.5
Contribution to Project:	Dr. Chitale is the official PI for this reporting period. He is aware and has approved Dr. Wang's management for this project. He has also completed the micro-dissection of the tumor FFPE specimens, directed the IHC analysis for the proteins, and directed DNA extraction <sup>3</sup> .
Funding Support:	<i>Employed in Henry Ford Hospital</i>

Name:	<i>Brandon Shaw PhD</i>
Project Role:	Sequence analyzer
Researcher Identifier (e.g. ORCID ID):	

Name:	<i>Brandon Shaw PhD</i>
Nearest person month worked:	<i>1</i>
Contribution to Project:	Dr. Shaw participated in the preliminary analysis of germline whole exome sequencing (WES) data from NF1 women affected with breast cancer. He used Omicia genome sequencing data analysis program.
Funding Support:	<i>NF Michigan, patient advocate organization</i>

○ **What other organizations were involved as partners?**

The genomic sequencing and analysis is ongoing in the genomic and biostatistic core in Moffitt Cancer Center. A Summary of all the prior and current collaborating and contracted institutions and personnel is list as below (the changes occurred in this report period is highlight in red):

**Primary study site (HFHS – Henry Ford Health System):**

Henry Ford Hospital, Pathology, 2799 W. Grand Blvd., K6, Detroit, Michigan 48202  
Dhananjay Chitale, MD PhD, **Current PI, previous Co-Investigator**

Henry Ford Health Systems, Biostatistics and Research Epidemiology, One Ford Place, Place 5C, Detroit, Michigan 48202  
 Statistical analysis  
 Next generation sequencing and WES manual analysis  
Albert Levin, PhD, collaborator

Moffitt Cancer Center, Genomics and Individualized Cancer Care, 10920 McKinley Drive, Office 5101, Tampa, Florida 33612  
Xia Wang, MD PhD, **Current consultant, previous PI**

**Subcontract clinical study sites:**

Johns Hopkins University School of Medicine, Department of Neurology, Brain Cancer Program, Cancer Research Bldg. II, 1550 Orleans Street, Ste. IM16, Baltimore, Maryland 21231  
JAISHRI BLAKELEY, MD, Co-PI

Children's National Medical Center, Jennifer and Daniel Gilbert Neurofibromatosis Institute, Department of Neurology, 111 Michigan Ave. NW, Washington DC, 20010  
Maria T. Acosta, MD, Co-PI



University of Alabama at Birmingham, Department of Genetics, 230 Kaul Human Genetics Bldg., 720 20th Street South, Birmingham, Alabama 35294  
Bruce Korf, MD, PhD, Co-PI

**Facility for tumor ion-torrent gene sequencing (This lab has discontinued providing this service. Therefore NGS is going to be completed by the genomic core lab in Moffitt Cancer Center)**

GeneDx Laboratory  
201 Perry Parkway, Suite 5  
Gaithersburg, MD 20877  
800-326-2685 ext. 8659  
1-301-990-2685 ext. 8659  
Adam Bennett, Executive Account Associate

**Facility for germline Whole Exome Sequencing (WES)**

Wayne State University, School of Medicine, C.S. Mott Center  
Applied Genomics Technology Center  
275 East Hancock  
Detroit, Michigan 48201  
(313) 577-6200  
Susan Land, PhD, Director of the core lab

**Tumor specimen next generation sequencing annotation program (HIPAA compliant)**

Omicia, Inc  
1625 Clay Street, 2nd Floor  
Oakland, CA 94612  
(510) 595-0800  
(510) 847-1046  
David Dailey, PhD

**Moffitt Cancer Center Genomic Core**

12902 USF Magnolia Drive, SRB building  
Tampa, Florida 33612  
Sean Yoder, MS

**Moffitt Cancer Center Biostatistic Core**

12902 USF Magnolia Drive, MRC building  
Tampa, Florida 33612  
Jamie Teer, PhD

## **8. SPECIAL REPORTING REQUIREMENTS**

Nothing to report

## 9. APPENDICES

### **The cancer occurrence, clinical features, and family histories of women with Neurofibromatosis type1 – A multi-center retrospective study**

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<sup>3</sup>Albert M. Levin: Henry Ford Health System, [Biostatistics & Research Epidemiology](#), Detroit, Michigan, U.S.A.

<sup>4</sup>Jaishri O. Blakeley: The Johns Hopkins Comprehensive Neurofibromatosis Center, Baltimore, Maryland, U.S.A.

<sup>5</sup>Maria T. Acosta: Gilbert Family Neurofibromatosis Institute, Children's National Medical Center, Washington D.C., U.S.A.

<sup>6</sup>Bruce R. Korf: Department of Genetics, Heflin Center for Genomic Sciences, University of Alabama at Birmingham, Birmingham, Alabama, U.S.A.

## **The cancer occurrence, clinical features, and family histories of women with Neurofibromatosis type1 – A multi-center retrospective study**

*Key words:* Neurofibromatosis type 1; breast cancer; clinical features; family history; central nervous system tumor

Word Count: 3,797

**Conflict of Interest:** None declared.

## Abstract

Neurofibromatosis type 1 (NF1) has a propensity to develop a variety of tumors. Despite the increased risk for malignant neoplasms and shortened life-span, there is no targeted cancer surveillance strategy. This multi-center retrospective study reviewed the records of 423 women with NF1. The associations between neoplasms, clinical features and family history were analyzed. The occurrence of breast cancers is positively associated ( $p=0.004$ ) with family history of any cancers, 9.6% (12/125) with family history vs. 2.7% (8/298) without. An association between NF1 clinical phenotypes (i.e. dermal neurofibroma burden) and cancer was not observed. However, the rate of malignant peripheral sheath tumor (MPNST) was significantly higher ( $p=0.049$ ) in women with plexiform neurofibroma (PN) than women without, 7.9% (11/139) vs. 3.14% (7/223). Women with learning disabilities have a higher rate ( $p=0.019$ ) of central nervous system (CNS) tumors including optic glioma (OPG) than women without, 22.2% (20/90) vs. 11.2% (21/187). European Americans (EAs) are significantly more likely ( $p=0.002$ ) to develop CNS tumors (21.2%, 41/193) than African Americans (AAs) (6.8%, 6/88).

## INTRODUCTION

Neurofibromatosis type 1 (NF1) is a pleiotropic autosomal dominant hereditary syndrome characterized by the occurrence of various types and numbers of benign and malignant neoplasms. The occurrence of gliomas, malignant peripheral nerve sheath tumors (MPNSTs), gastrointestinal stromal tumors (GISTs), and pheochromocytomas is significantly elevated compared to the general population.[1,2] The rate of colon and breast cancers are moderately increased, especially among individuals 50 years or younger.[3-7] A hospital admission based record-linkage population study has also shown an elevated risk for other common cancers, such as liver, esophagus, stomach, pancreas, biliary tract, lung, skin, thyroid, ovarian, leukemia and lymphoma in people with NF1.[8] The spectrum of non-neoplastic clinical and physical features of NF1 is also wide. Despite the increased risk for malignant neoplasms, there is no established

protocol to screen for cancer in people with NF1 beyond the guidelines for general population. If any clinical features of NF1 and/or family history are found to be associated with occurrence of certain neoplasms, these may serve as indicators for targeted cancer or neoplasm surveillance. This multi-center case review study was designed to explore the associations between the occurrence of neoplasms and the physical/clinical features of NF1 in women with NF1. The overall goal is to identify factors associated with breast cancer in women with NF1.

## **METHODS**

### **Study Subjects**

Comprehensive medical record review was conducted in three Children's Tumor Foundation (CTF) affiliated neurofibromatosis clinics in the United States: Henry Ford Health System (HFHS), University of Alabama at Birmingham (UAB), and Johns Hopkins University (JHU). Children's National Medical Center (CNMC) in the District of Columbia also recruited and collected medical information from affected mothers whose children were evaluated in the NF clinic. The medical records were reviewed for all females 20 years or older at the time of study, who either meet the clinical diagnostic criteria of NF1[9] or carry a deleterious mutation in the NF1 gene. The four hundred and twenty three cases collected include all women who were seen in the clinic during the following periods of time: 114 cases (1994 to 2013) in HFHS, 122 cases (2011 to 2013) in UAB, and 156 cases (2003 to 2013) in JHU. In CNMC, 31 cases were collected from 2011 to 2013.

### **Data Collection**

Demographic information gathered included date of birth, ethnicity, and biological relationships within the cohort. Medical information gathered included clinical features, such as the number of café-au-lait macules on the skin, presence of skin fold freckling, Lisch nodules on the irises, bony dysplasia, macrocephaly, short stature and learning disability. Neoplasm-specific information collected includes the number of cutaneous neurofibromas, plexiform neurofibromas (PN), optic gliomas (OPG), malignant peripheral nerve sheath tumor (MPNST), as well as other malignant solid tumors, malignant hematological disorders, malignant or benign tumor of the central nervous system (CNS). OPG is a tumor originated from neural glial astrocytes. It develops on the tract of optic nerve during the first several years in life. In this report, it is discussed as a separate entity from other CNS tumors.

For women identified as having breast cancer, the histological type, stage and age at diagnosis were recorded when available. Breast cancer screening and breast biopsy information was also collected. Family history information gathered included NF1, malignant neoplasm, CNS tumor, and the number of relatives with breast cancer. Genetic test results such as *NF1* gene mutation and/or *BRCA1* and *BRCA2* mutation were documented when available. The occurrence of malignant neoplasms and CNS benign or malignant tumors were assessed for their possible association with clinical features associated with NF1. The CNS tumor category includes all tumors, from low grade glioma to high grade glioblastoma. A feature of thickened optic nerve or chiasm was not counted as OPG. The source of information and clinical features documented in the medical record was either self-reported by the patients or supported by clinical evidence.

## **Statistical Analysis**

While an attempt was made to collect complete data on all subjects, the validity of multivariate analysis was limited due to missing data. Therefore, we have restricted the presentation of results to only those from the univariate analyses, assuming that the data for individual variables are missing completely at random. We used Fisher's exact tests to evaluate the statistical significance of association between each discrete clinical feature and prevalent cancers. P-values less than 0.05 were considered to be statistically significant. For ease of interpretability, odds ratios (OR) and corresponding 95% confidence intervals were also estimated to provide estimates of effect.

## **RESULTS**

A total of 423 cases of women affected with NF1 were reviewed. The study sample comprised 250 European Americans, 118 African Americans, and 41 individuals of other ethnicities. Ethnicity information was not available for 14 women. Among all, 36 women are related to at least one other woman in this cohort and belong to a total of 16 kinships (Table 1). Family history of NF1 in female relatives was collected based on the pedigree in chart. At least one female relative was affected with NF1 for 162 women. There were no female relatives affected with NF1 for 215 women. The status of family history of NF1 was not available for 46 women.

At least one type of cancer was reported in 98 women with NF1. Nineteen of them have had at least two primary cancers. The breakdown of observed neoplasms is presented in Table 2. There were 205 prevalent cancer/neoplasms in the relatives of 125 women with NF1. These included 9 NF1 related cancers (consisting of brain tumor and MPNST), 4 neuroendocrine tumors (consisting of pheochromocytoma and pituitary tumor), 4 sarcomas, 8 hematological cancers, 75 breast cancer, and 105 other cancers (consisting of 21 lung, 18 colorectal, 4 esophageal, 2 gastric, 6 head and neck, 3



cervical, 5 ovarian, 3 uterine, 3 bladder, 12 prostate, 3 renal, 9 skin or melanoma, 3 pancreatic, 3 thyroid, 6 “bone”, 2 “thoracic” cancer and 1 metanephric stromal tumor).

**Breast cancer:** Of the 20 women who have a personal history of breast cancer, 15 were previously reported by Wang [5] and Madanikia [6] in 2012. Eleven are European Americans and 8 are African Americans. Ethnicity information for the remaining individual is not available (Table 3). None of these women is known to be genetically related to one another. Half of the cases (n=10) were diagnosed with breast cancer between the age of 40 to 49 years. A quarter of the cases (n=5) were diagnosed between 30 to 39 years of age. Two cases were diagnosed with a second primary breast cancer. All of these breast cancers are ductal carcinoma, except one invasive lobular carcinoma, which is estrogen receptor (ER) positive (Table 4). Only one case was known to be an ER-PR (estrogen-progesterone receptor) negative and HER2 expression negative (i.e. triple negative) invasive ductal carcinoma. Two cases are known to be ER-negative, HER2 expression positive tumors.

**Breast cancer and family history:** The prevalence of personal history of breast cancer was nearly four-fold higher (odds ratio OR=3.83, 95% confidence interval 95%CI=1.40-11.12) for women with NF1 and a family history of any cancers (9.6%; 12/125) in comparison to those without a family history (2.7%, 8/298), which was statistically significant (p=0.004). The type of cancers in the family history does not differ significantly between the women with breast cancer and those without. However, when there is a family history of 3 or more cancers, the rate of personal breast cancer is 4 times higher (26.3% 5/19) than the rate when there are only 1 or 2 cancers in the family (6.6% 7/106), p=0.019. The prevalence of personal breast cancer in the presence of a family history of breast cancer in 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> degree

female relatives (10.7%, 8/75) is more than 3-fold higher (OR=3.46; 95% CI=1.09 – 11.02) than in the absence of a family history (3.3%, 8/241), which is statistically significant ( $p=0.029$ ). However, breast cancer is not significantly associated with family history of female relatives with NF1 ( $p=0.434$ ). In this cohort, none of the women with breast cancer had a reported family history of any relative affected with NF1 and breast cancer.

**Breast Cancer and Clinical Features of NF1:** For cases with available clinical features, statistical analysis has not detected any association between the NF1 features, breast cancer or other cancers (all  $p \geq 0.16$ , Supplementary table 1). It is noteworthy to mention that high cutaneous neurofibroma burden (20 or more or described as “diffuse” documented in the medical record) is not significantly associated with any types of cancer ( $p=1.00$ ).

**Breast Cancer Screening Status:** The information for breast cancer screening is available for 31.4% (133/423) of the cases. Among them, 59.4% (79/133) are reported to have had physical breast examination, 33.1% (44/133) have a history of breast lumps, 23.3% (31/133) underwent biopsy of the breast. For breast imaging evaluations, 48.1% (64/133) had mammogram. Although breast neurofibromas are recorded in 10% (42/423) of the women in this cohort, only 3.76% (5/133) of cases underwent MRI evaluation for breast lumps.

**MPNST and Plexiform Neurofibroma:** The occurrence of MPNST is related to plexiform neurofibromas (PN). Among women with documented PN, 7.9% (11/139) have a history of MPNST, which is significantly higher ( $p=0.049$ ) than the women without documented PN, 3.14% (7/223).

**CNS tumor, optic glioma (OPG) and learning disability:** The prevalence of CNS tumors (excluding OPG) is nearly 6-times higher (OR=5.73, 95%CI=1.54 – 20.13) in women with a history of OPG (14.6%, 6/41) in comparison to the women without OPG (2.9%, 8/278), which was statistically significant (p=0.004). The women with learning disability have a 2.25-fold (95%CI=1.08-4.67) higher rate of CNS tumor, OPG or both (i.e. “CNS+OPG”) (22.2%, 20/90) than those without a learning disability (11.2%, 21/187), which is significant (p= 0.019). Due to the small number of cases, the relationship between learning disability and CNS tumor excluding OPG cannot be determined at this time. However, upon exclusion of the cases with CNS tumor alone, the association between learning disability and OPG with or without CNS tumors is suggestive, but not statistically significant in this cohort, 16/90 vs. 19/187, p=0.083.

**Ethnicity and Malignant Neoplasms:** The rate of “CNS+OPG” and “Other cancers” varies significantly by ethnicity. “Other cancers” refers to all malignant tumors, hematological malignancies, CNS tumors and OPG, excluding breast cancer. For the “CNS+OPG” category, European Americans (EAs) were 3.72 times (95% CI=1.48 – 11.16) more likely to develop these tumors (21.2%, 41/193) than African Americans (AAs) (6.8%, 6/88), which was statistically significant (p=0.002). The occurrence of OPG with or without CNS tumor is also higher (OR=3.48, 95%CI=1.28 – 11.88, 95%) in EAs (17.4%, 32/184) than AAs (5.7%, 5/88), which was significant (p=0.008). For the “Other cancers” category, EAs were also significantly (p=0.004) more likely to develop these tumors (26.8%, 67/250) than AAs (13.5%, 16/118). Analysis could not demonstrate a statistically significant association between ethnicity and breast cancer (p=0.301).

**Ethnicity and other clinical features:** Lisch nodules are more common in EAs (59%, 100/170) relative to AAs (39%;  $p=0.009$ , 26/66) or other ethnicities (32%;  $p=0.010$ , 10/31). There is also a significant difference between the number of individuals with higher dermal neurofibroma burden, i.e. 20 or more or described as “diffuse” at the time of clinical evaluation, by ethnicity, with AAs having a higher rate (75.8%, 47/62) of high tumor burden than EAs (53.0%, 70/132;  $p=0.003$ ).

## DISCUSSION

Previous studies have revealed a significantly elevated breast cancer risk, 4-8 fold, in women with NF1 under age 50 in England and the United States.[3-7] For women age 50 or older, the risk is also elevated, but to a lesser degree, 1.9-2.6 fold. This phenomenon leads to the suspicion that a pathogenic germline NF1 genetic variant may be an independent risk factor for breast cancer. Based on this assumption, family history of NF1 should be associated with breast cancer in this population. This hypothesis is not supported by the results of this study, however. This study demonstrates that a personal history of breast cancer in women with NF1 is associated with a family history of breast cancer ( $OR=3.46$ ) or all cancers ( $OR=3.83$ ) while it does not appear to be solely dependent on family history of NF1 in female relatives, or perhaps the magnitude of dependence is not strong enough to be manifested in this study. Population study based on Swedish database has characterized the breast cancer risk elevation associated with family history of breast cancer in first and second degree relatives. The relative risk (RR) was 1.27 when a sister was affected. The RR was 1.74 when a mother was affected. The RR was 2.8 when at least 2 first degree female relatives were affected.[10] This association appears to be at a lesser degree than our cohort of NF1, although the NF1 women’s family history included all first, second and third degree

relatives. It was not stratified based on the number of the affected relatives either. The observation in NF1 women suggests that these breast cancers are possibly the results of environmental exposure shared by the family members, or the results of additional underlying germline cancer predisposition genetic variants, or a combination of both. Therefore, the higher prevalence of breast cancer in NF1 is likely a manifestation of synergistic effects when individuals with an NF1 gene mutation encounter environmental carcinogens or other hereditary cancer predisposition genomic variants. The pattern of cancers in the family history shows no significant difference between the women with breast cancer and the women without, thus offers no clue as what sort of carcinogens or hereditary genomic predispositions may have contributed to this synergistic effect. Exploration of the co-occurring germline genomic mutations or variants may provide further clues. Germline Whole Exome Sequencing (WES) analysis for NF1 women affected with breast cancer will soon be completed by Wang and colleagues. Based on the current data, it is promising to use family history of breast cancer or any cancers to serve as a risk indicator for personal breast cancer in women affected with NF1. This risk association is even stronger as the number of cancers in the family increases. These women affected with breast cancer may have inherited other cancer predisposing genetic variants.

The distribution of the histological types and hormonal receptor status for the breast cancers in women with NF1 does not differ significantly from the general population, except ER-negative tumors are under represented. Another manuscript will explore this in detail.

This study has shown that even with the recognized risk, breast cancer screening and breast lump evaluation are not adequate for women with NF1. Among the patients with available information,

approximately 23% NF1 women underwent biopsy for the lumps in the breast, and 10% were known to have neurofibroma within the breast. Clinical experience tells us that physical examination with palpation or mammogram cannot reliably distinguish a neurofibroma from a malignant breast nodule. Dedicated breast MRI with gadolinium would be more descriptive as it will characterize and enhance the well encapsulated neurofibroma within the parenchyma. Nearly 85% of the breast carcinomas are non-capsulated. Our data suggests that a majority of the breast lumps (70%, 31/44) were further evaluated by biopsy. However, only 5 cases were evaluated by MRI. Future research assessing breast MRI for cancer screening and nodule assessment may provide better evidence.

The association between MPNST and plexiform neurofibroma supports the previous evidence that the majority of MPNSTs emerge from preexisting plexiform neurofibroma.[1] Therefore, preexisting PN may also serve as a risk indicator for MPNST.

Our study suggests that OPG during childhood may serve as a risk indicator for future occurrence of brain tumor. However, this study was not designed to study the timing of diagnosis or the character or treatment of OPG. Therefore, we cannot exclude the possibility that the following two factors have at least partially contributed to the association: 1) Asymptomatic OPGs were discovered during brain tumor evaluation; 2) Radiation therapy for OPG induced the CNS tumor later in life. An increased rate of CNS tumors later in life was previously reported among patients who have had radiation therapy for OPG.[11] The current standard is to avoid using radiation therapy for OPG in individuals with NF1. Our study also demonstrated an association between learning disability and CNS and/or OPG tumor, which suggests a common defect hindering the CNS development congenitally as well as predisposing to CNS

tumor formation later in life. In individuals with NF1, OPG mostly occurs during early childhood. As treatment for OPG, chemotherapy or radiation is known to have adverse effects on the developing brain, leading to learning disability. In a recent 20 year-perspective study of OPG, 149 children were diagnosed with OPG by MRI screening. Only 22 children required treatment.[12] Nevertheless, learning disability as a side effect of OPG treatment and/or a large tumor altering brain function could have partially contributed to the association between these two variables. More advanced study with information regarding OPG treatment, as well as metrics assessing learning disability before and after CNS or OPG treatment will allow us to better characterize the relationships.

Our study shows that predisposition to CNS tumors and/or OPG is disproportionally higher in European Americans, in comparison to African Americans. A prior report of smaller sample size in 1998 suggested the predilection for OPG in EAs versus AAs.[13] A recent larger cohort retrospective study has also demonstrated this phenomenon.[12] This trend coincides with the observations that the incidence of sporadic malignant CNS tumor in non-Hispanic whites is around 2-4 times that of the blacks in North America.[14,15] All of above suggests that the CNS tumor or OPG in NF1 patients may share a common pathway in tumorigenesis as sporadic brain tumors. As such, European American ethnicity is also a risk indicator for brain tumor in the population with NF1. Based on 2010-2012 SEER (Surveillance, Epidemiology, and End Results Program in National Institute of Health, United States) data, approximately 0.6 percent of men and women in the general population will be diagnosed with brain and other nervous system cancer at some point during their lifetime. Since the baseline occurrence of brain tumor in NF1 is increased 22 fold,[3] brain tumor screening may be beneficial for EAs with NF1 if the lifetime risk is proven significant by larger cohort study.

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**Contributorship Statement:**

Xia Wang has initiated and designed the entire project. She has written the manuscript.

Renee N. Tousignant has participated in the design of the questionnaire, coordinated the data collection and contribution among collaborating centers. She also analyzed part of the data set. She has reviewed and contributed to the manuscript.

Albert M. Levin participated in the initial planning and designing of this project. He provided important constructive recommendations. As an experienced biostatistician, he has also recommended and employed the proper statistical methods for data analysis. He has reviewed and contributed to the manuscript.

Jaishri O. Blakeley participated in the initial planning as a collaboration site PI. She has ensured the proper data collection and review in her institution (Johns Hopkins), as well as compliance with the privacy and ethnical policies. She has reviewed and contributed to the manuscript.

Maria T. Acosta participated in the initial planning as a collaboration site PI. She has ensured the proper data collection and review in her institution (Gilbert Family Neurofibromatosis Institute,



Children's National Medical Center), as well as compliance with the privacy and ethical policies. She has reviewed the manuscript.

Bruce R. Korf participated in the initial planning as a collaboration site PI. He has ensured the proper data collection and review in his institution (University of Alabama at Birmingham), as well as compliance with the privacy and ethical policies. He has provided critical review, recommendations and guidance for the interpretation of data. He has reviewed and contributed to the manuscript.

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